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Intragenomic Complementation of a 3AB Mutant in Dicistronic Polioviruses

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We report the construction of a poliovirus genome [pPVM-VPg(3F4A)] harboring a double mutation in VPg. This mutant, in which the tyrosine and the threonine at residues 3 and 4 of the VPg region were replaced by phenylalanine and alanine, respectively, is lethal, that is, all RNA synthesis was abolished and no revertants could be isolated. Using the properties of dicistronic polioviruses (with the general genotype *PV* 5′NTR-3AB-*EMCV* IRES-*PV* ORF-3′NTR), we have observed that the defect in RNA synthesis of the VPg(3F4A) mutant could be rescued by providing wild-type protein 3AB from the first open reading frame *in trans.* We conclude that the 3AB provided by the first cistron of the dicistronic construct was capable of "intragenomic complementation." Intragenomic complementation, however, was inefficient. Thus, the dicistronic RNAs were only quasi-infectious, and even first-passage viruses were found to have reverted to a functioning VPg in the polyprotein. This phenomenon underlines the role of polypeptide 3AB in multiple functions of viral proliferation. First-passage viruses, all of which expressed a small-plaque phenotype, had retained the original dicistronic genotype. At the fourth passage, however, all isolates were monocistronic, and they displayed complex genetic rearrangements revealing interesting information regarding IRES function.

INTRODUCTION

Poliovirus encodes only 1 polypeptide, the polyprotein (Fig. 1A; Kitamura *et al.,* 1981; Racaniello and Baltimore, 1981). Under physiological conditions, the polyprotein is highly unstable and cleaves itself *in statu nascendi* into primary products that are subject to further cleavage events. Altogether, poliovirus may produce as many as 27 different polypeptides by proteolytic processing (Harris *et al.,* 1990). Some of the viral polypeptides are not only precursors in the cascade of cleavages, but they are proteins with functions distinct from that of their cleavage products, a phenomenon particularly relevant to the products of the P3 region of the polyprotein (Andino *et al.,* 1993; Flanegan and Baltimore, 1977; Hanecak *et al.,* 1982; Harris *et al.,* 1994; Paul *et al.,* 1994; Semler *et al.,* 1982; Takegami *et al.,* 1983; Ypma-Wong *et al.,* 1988). Moreover, a single viral protein, as an end product of proteolytic processing, may serve numerous distinct functions. A striking example of such a multifunctional protein is the viral proteinase $2A^{pro}$ (Bernstein *et al.,* 1986; Hambidge and Sarnow; 1992; Lamphear *et al.,* 1993; Molla *et al.,* 1993; Toyoda *et al.,* 1986).

Genetic complementation in poliovirus replication has been widely documented (Wimmer *et al.,* 1993). It has not been possible, however, to establish complementation groups. This is most likely related to the peculiar mechanism by which viral proteins encoded in the P2–P3 region exhibit a multitude of different functions inherited by a single polypeptide (Wimmer *et al.,* 1993). These genetic results together with the unique gene organization of the virus have led to the suggestion that, *de facto,* the poliovirus genome must be viewed as being monocistronic (Wimmer *et al.,* 1993).

Mutations mapping to different regions of the polyprotein may be recessive (complementable) or *cis*-dominant (noncomplementable). So far, all mutants mapping to 2B have been found to belong to the latter category (Johnson and Sarnow, 1991). Interestingly, mutations mapping to the coding region of 3A and $3D^{pol}$ may either be recessive or be *cis*-dominant (Agut *et al.,* 1989; Bernstein *et al.,* 1986; Charini *et al.,* 1991; Giachetti *et al.,* 1992; Trono *et al.,* 1988). It has yet to be established, however, whether the mutations in the polypeptides cited above exert their effect on the function of the precursors 2BC, 3AB, or $3CD^{pro}$, or on the function of the cleavage products 2B, 3A, or $3D^{pol}$, respectively. A special case is unidirectional complementation, that is, the rescue of a mutant virus by a superinfecting wild-type virus. Rescue applies, for example, for the encapsidation of poliovirus DI particles (reviewed in Wimmer *et al.,* 1993).

Studies on poliovirus polypeptide 3AB have suggested that this protein is involved in several different processes crucial for viral replication. 3AB is considered to be the precursor for VPg, the genome-linked protein (Semler *et al.,* 1982; Takeda *et al.,* 1986; Takegami *et al.,* 1983). Due to an extensive hydrophobic region within the coding sequence of 3A, most of 3AB is membrane-associated in infected cells (Semler *et al.,* 1982; Datta and Dasgupta, 1994), and cleavage of 3AB to 3A and VPg by $3CD^{pro}$ *in vitro* requires this hydrophobic environment (Lama *et al.,*

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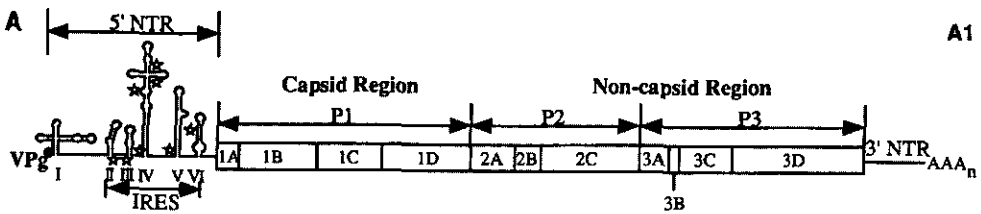

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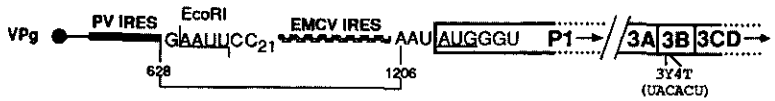

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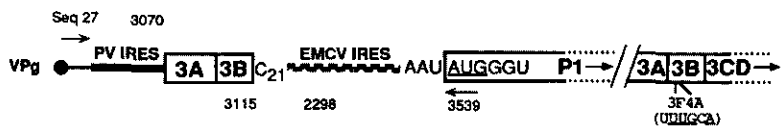

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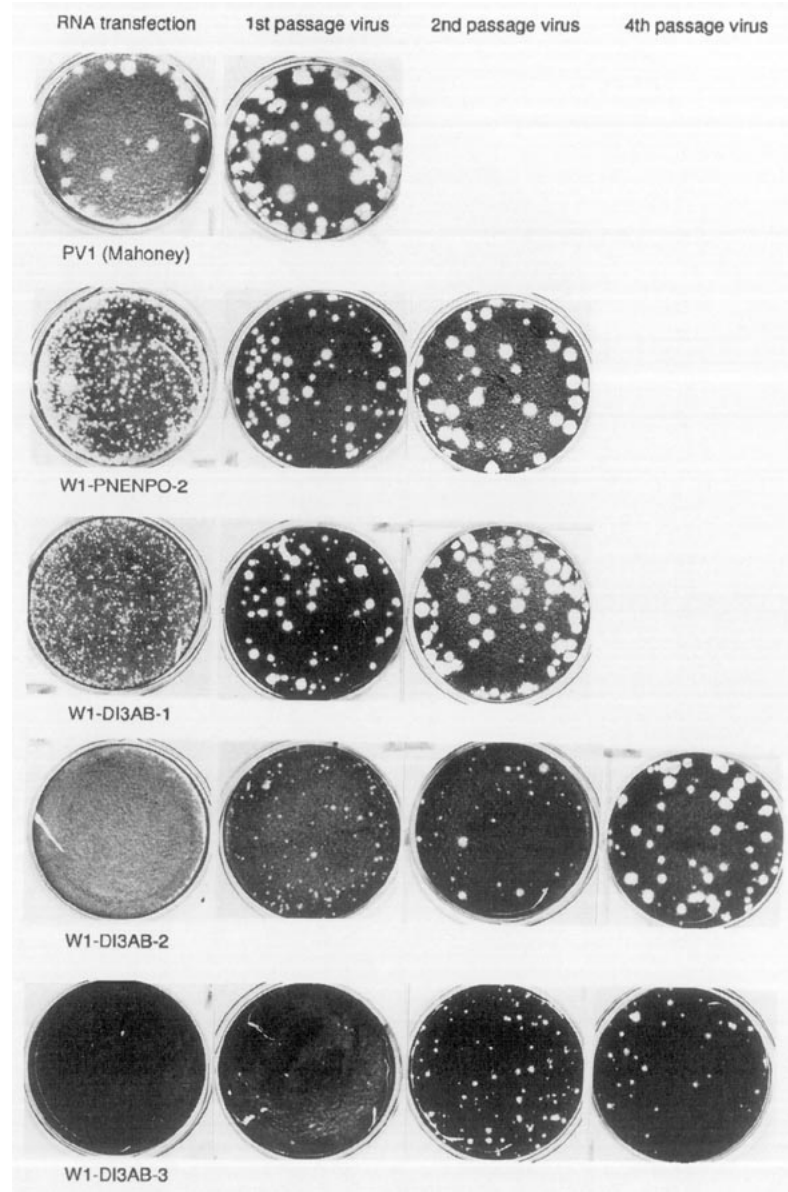

B

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   G  A  Y  T  G  L  P  N  K  R  P  N  V  P  T  I  R  T  A  K  V  Q
3B GGAGCAUACACUGGUUUACCAAACAAGAGGCCUAACGUGCCCACCAUUCGGACCGCAAAGGUACAA
3B --------U--A--AC-U--U--U--A--A--A--U--C--A--A--CA-A--A--U--A--U--G
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1994). Moreover, several mutations engineered into the hydrophobic region of 3AB have shown lethal or severely impaired replication phenotypes (Giachetti *et al.,* 1992). VPg (=3B) is linked to the viral RNA via a phosphodiester bond between the 5′-terminal uridine residue of the genome and the O^4 hydroxyl group of its single tyrosine residue (Fig. 1B) (Rothberg *et al.,* 1978; Ambros and Baltimore, 1978). Several mutations mapping to VPg also expressed a lethal phenotype, including a mutant in which the tyrosine at residue 3 was changed to phenylalanine (Kuhn *et al.,* 1988a,b; Reuer *et al.,* 1990). Available evidence supports a model in which the uridylylated form of VPg (or 3AB) may serve as primer for $3D^{pol}$ (Takeda *et al.,* 1986; Takegami *et al.,* 1983; Toyoda *et al.,* 1986). Alternative mechanisms for the formation of the Tyr–O^4–pU linkage have been proposed (Tobin *et al.,* 1989).

3AB is also a nonspecific RNA binding protein (Paul *et al.,* 1994) that has the propensity to stimulate the polymerase activity of $3D^{pol}$ up to 100-fold (Lama *et al.,* 1994; Paul *et al.,* 1994). 3AB and the proteinase $3CD^{pro}$, two proteins mapping adjacent to each other (Fig. 1A), form a complex in solution that leads to the enhanced rate of proteolysis of $3CD^{pro}$ and $3D^{pol}$ (Molla *et al.,* 1994). Apart from the mutual effects with regard to proteolytic processing, the 3AB/$3CD^{pro}$ pair has been found to bind strongly to the 5′-terminal cloverleaf of the viral genome (Harris *et al.,* 1994), a phenomenon that appears to be essential for genome replication (Xiang *et al.,* 1995). It remains to be elucidated whether a cellular protein (p36) that is able to facilitate the binding of $3CD^{pro}$ to the cloverleaf is essential for genome replication (Andino *et al.,* 1993, Harris *et al.,* 1994). Recently, Datta and Dasgupta (1994) have described that a portion of both 3AB and 3A expressed *in vitro* can be glycosylated, a phenomenon the biological significance of which is not yet known.

We have recently constructed a viable dicistronic (*dc*) poliovirus (W1-DICAT) that carries a foreign gene (chloramphenicol acetyl transferase, or CAT) in the 5′-terminal region between two internal ribosomal entry sites (IRES) of poliovirus (PV) and encephalomyocarditis virus (EMCV) (Alexander *et al.,* 1994). W1-DICAT, whose genotype is *PV* 5′NTR-CAT-*EMCV* IRES-*PV* ORF-3′NTR, has been found to efficiently express the CAT gene over several viral passages (Alexander *et al.,* 1994). This observation has offered the intriguing possibility to design a *dc* virus in which the CAT was exchanged with the coding region of a poliovirus protein and to test this construct for its ability to complement a defect in the second cistron, the polyprotein. In view of the apparent complex functions of 3AB in poliovirus replication, we have targeted 3AB for this experiment. Accordingly, we have constructed a *dc* poliovirus genome with the genotype *PV* 5′NTR-3AB*wt-EMCV* IRES-*PV* ORF-3′NTR that contained a lethal mutation in 3B (=VPg) in the polyprotein. Transfection of HeLa cells with the corresponding RNA yielded viable viruses, a result that can only be explained if the *wt* 3AB in the first cistron was capable of intragenomic complementation (rescue) of the defective 3AB in the poliovirus polyprotein. Passages of the dicistronic viruses have led to the complete deletion of the first cistron. We have also observed that the natures of the genetic rearrangements were significantly changed when the genotypes of the two 3B sequences in a *dc* virus were constructed to be heterologous.

MATERIALS AND METHODS

Construction of pPVM-VPg(3F4A)

To generate double amino acid substitutions at positions 3 and 4 in VPg region of the poliovirus genome, two-step polymerase chain reactions (PCR) were performed. Oligonucleotides 2910 (5′-GGGTTGGATAGTTAACATCACCAGCC-3′, plus-strand, nucleotide positions 5230–5255) and 2912 (5′-CCTGCTTGATCTTCGAGCGC-3′, complementary to nucleotide positions 5618–5637) were used as external primers; oligonucleotides Seq08 (5′-CCAGGGAGCATTTGCAGGTTTACC-3′, plus-strand, nucleotide positions 5368–5391) and Seq10 (5′-TGCAAATGCTCCCTGGTGACC-3′, complementary to nucleotide positions 5363–5383) were used as internal primers. Seq08 and Seq10 contained nucleotide changes at positions 5378–5383 (underlined), which resulted in the replacement of amino acids tyrosine and threonine to phenylalanine and alanine, respectively. First, two PCR

FIG. 1. Schematic depiction of the dicistronic constructs and plaque assays. (A1) pT7-PVM, a plasmid encoding the full-length PV1 cDNA. Secondary structure represents the cloverleaf and the IRES elements as indicated, and the nomenclature of structural elements of domains I to VI is according to Wimmer *et al.* (1993) (structures not drawn to scale). Stars indicate positions of AUG triplets. The open box depicts the polyprotein ORF, starting at nt 743, which is divided into domains P1, P2, and P3. (A2) pPNENPO-2, showing only the 5′-NTR. The *Eco*RI restriction site is inserted between PV and EMCV IRES elements. The number 628 represents the position in the poliovirus genome and 1206 represents the position in the dicistronic construct pPNENPO-2 where the EMCV IRES is inserted; the initiating AUG is underlined. Wild-type amino acids 3Y4T in the 5′-end of 3B (VPg) region is indicated (the coding nucleotide sequences are shown in parentheses). (A3) pDI3AB-1, both 3AB gene units at the 5′-end and the 3′-end of the genome are wild type. (A4) pDI3AB-2, the 3AB gene at the 5′-end of the genome is wild type and the 3AB coding unit at the 3′-end contains the amino acid mutations 3F4A (the coding nucleotide sequences differing from the wild type are underlined in the parentheses). (A5) pDI3AB-3, similar to pDI3AB-2, except that the VPg region in the 5′-3AB gene is heterologous (see B). Primers used for RT–PCR are indicated above and below diagrams of A4 and A5. Arrows indicate the polarity of the primers. (B) Amino acid sequence and the coding nucleotide sequences in the VPg region. The open box represents the wild-type nucleotide sequence of 3B (VPg), the shaded box represents the heterologous nucleotide sequence of 3B in which only the nucleotides that differ from the wild type are shown, and the nucleotides identical to the wild type are aligned. Plaques are produced by the viruses that originated from transcript RNAs of the corresponding plasmids. All plaques were developed after 48 hr incubation at 37°, except for the first-passage virus derived from pDI3AB-3 (72-hr incubation at 37°).

reactions were performed using the VPg mutagenesis cartridge pT7-VPg15 (Kuhn *et al.*, 1988a) cDNA as template for extension of the primers 2910 and Seq10 in one reaction and of the primers Seq08 and 2912 in another reaction. In the second step, the two resulting PCR fragments were mixed and used as templates in a new PCR reaction with the primers 2910 and 2912. The final product (404 bp) was digested with *Hpa*I–*Bgl*II to yield a 363-bp fragment, which was then inserted into the poliovirus cDNA clone pT7-PVM (Cao *et al.*, 1993) at the *Hpa*I–*Bgl*II site. After selection and propagation in the *Escherichia coli* strain DH5, the recombinant plasmid pPVM-VPg(3F4A) was confirmed by sequence analysis in the region of mutagenesis.

Construction of subclones pPVM-5′ (EMCV), pPVM-5′ (DI3AB)-1, and pPVM-5′ (DI3AB)-3

In order to create unique restriction enzyme digestion sites *Spe*I and *Eco*RI in the 5′-NTR of the poliovirus genome, the full-length cDNA clone pT7-PVM was digested with *Spe*I–*Eco*RI, yielding three fragments with lengths 1341, 3641, and 5727 bp. The 5727-bp fragment that contained the pBR322 vector and the 5′-end of poliovirus genome (up to position 2641) was filled in with T4 DNA polymerase and religated together. This newly generated subclone pPVM-5′ (0–2641) was used for the construction of further plasmids.

Construction of pPVM-5′ (EMCV). An *Eco*RI–*Bst*BI-digested fragment was obtained from W1-PNENPO (Alexander *et al.*, 1994), which contained the entire EMCV IRES element and the truncated P1 region (up to position 865) of poliovirus genome. After the *Eco*RI end was filled in with T4 DNA polymerase, the *Eco*RI–*Bst*BI fragment was inserted into the *Msc*I–*Bst*BI region of the cDNA clone pPVM-5′ (0–2641) to generate the required construct pPVM-5′ (EMCV). The resulting construct contained the EMCV IRES inserted between the poliovirus IRES and the open reading frame. A unique *Eco*RI site was located at the very 5′-end of EMCV IRES.

Construction of pPVM-5′ (DI3AB)-1 and pPVM-5′ (DI3AB)-3. Wild-type 3AB fragment was generated by PCR using pT7-VPg15 cDNA as template, with oligo pair 3113 (5′-ATGGGACCACTCCAGTATAAAGAC-3′, plus-strand, at the 5′-end of 3A) and 3115 (5′-ATTGTACCT-TTGCGG-3′, complementary to the 3′-end of 3B) as primers. After phosphorylation treatment with T4 polynucleotide kinase, the 3AB fragment was then ligated into pPVM-5′ (EMCV) that had been digested by *Eco*RI and treated with the Klenow enzyme. This construct pPVM-5′ (DI3AB)-1 contained a start codon ATG and a stop codon TAA at the 5′-end and the 3′-end of 3AB region.

pPVM-5′ (D3AB)-3 was generated similarly to pPVM-5′ (DI3AB)-1, except that the wild-type 3AB fragment was derived from a cDNA clone pPVM-rV10 (construction to be published elsewhere). In pPVM-rV10, the nucleotide sequence in the 3B region was substituted at all the third position and some of the second position of most of the codons without changing the coded protein sequence (as shown in Fig. 1B, the shaded box 3B); transfection of this RNA onto HeLa monolayers produced virus plaques with wild-type size (unpublished data).

Construction of W1-PNENPO-2, W1-DI3AB-1, W1-DI3AB-2, and W1-DI3AB-3

The subclones pPVM-5′ (EMCV), pPVM-5′ (DI3AB)-1, and pPVM-5′ (DI3AB)-3 were digested with *Nhe*I–*Fsp*I to yield either a 5550- or a 5883-bp fragment. Fragments derived from pPVM-5′ (EMCV) or pPVM-5′ (DI3AB)-1 were inserted into the *Nhe*I–*Fsp*I region of the full-length cDNA clone pT7-VPg15 to generate constructs W1-PNENPO-2 or W1-DI3AB-1, respectively. Fragments derived from pPVM-5′ (DI3AB)-1 or pPVM-5′ (DI3AB)-3 were inserted into the *Nhe*I–*Fsp*I region of the full-length cDNA clone pPVM-VPg (3F4A) to generate construct W1-DI3AB-2 or W1-DI3AB-3, respectively (see Fig. 1A).

In vitro transcription, RNA transfection, and virus plaque assay

Transcription of RNA by T7 RNA polymerase was performed as previously described (Cao *et al.*, 1993). RNA transfection of HeLa cell (R19) monolayers using DEAE-dextran was performed as previously published (Kuhn *et al.*, 1988b). Direct agar overlay of RNA transfection for phenotype analysis was performed as described (Cao *et al.*, 1993). For plaque assays, virus stocks were derived either from total cell lysates of RNA transfections or from single plaque-purified viruses. Monolayers of HeLa cells were first incubated with the diluted virus solutions in PBS at room temperature for 30 min and then overlayed with DMEM-agar containing 5% fetal bovine serum. The monolayers were further incubated at 37° until plaques were formed.

Viral RNA Sequencing and reverse transcription–PCR (RT–PCR)

HeLa cell monolayers (4×10^6 cells) were infected with viruses that were obtained from RNA transfections and subsequent virus reinfections with or without plaque purification, at a multiplicity of infection of 10. At 7 hr postinfection, total cytoplasmic RNA was extracted as described previously (Reuer *et al.*, 1990). Different regions of the RNA were sequenced by the dideoxy chain-termination method using α-^{35}S-dATP (Amersham) and avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim).

For RT–PCR, the RNAs purified from virus infections were reverse transcribed at different higher temperatures (60–72°) using Tth DNA polymerase (Boehringer-Mannheim) and oligonucleotides 3115, C14, 2298, and 3539 as primers (see below). After treatment of the synthesized cDNAs with RNase A, PCR was performed using these cDNAs as templates and oligonucleotides 3115 (as

above), C14 (5′-ACTGAACTTTAGCTGTTCTG-3′), 2298 (5′-GACAAACGCACACCGGCC-3′), 3539 (5′-GCGCCC-ACTTTCTGTGATGAAACC-3′), Seq27 (5′-TTAAAACAG-CTCTGGGGTTG-3′), and 3070 (5′-GTGGCTGCTTATG-GTGAC-3′) as primers. The positions of these oligos are shown in Figs. 1A4 and 1A5.

In vitro translation and product analysis

HeLa cell extract was prepared, treated with micrococcal nuclease, and used for translation as described previously (Molla *et al.*, 1991). Viral RNAs transcribed *in vitro* were mixed with a nuclease-treated cell extract plus translation mixture (containing essential ingredients for translation) and 1 mCi of [α-^{35}S]methionine (ICN Radiochemicals)/ml. Incubations were performed at 34° from 30 min to 8 hr. Samples were analyzed by a 10–20% gradient SDS–polyacrylamide gel electrophoresis. Gels were fixed, treated with En^{3}Hance (ICN Radiochemicals), dried, and exposed to X-ray film for 24 or 48 hr at −70°.

RESULTS

Analysis of the *dc* constructs by *in vitro* translation

The *dc* viruses employed here were constructed by insertion of the extra gene into a genome containing two tandemly arranged IRES elements in the 5′-NTR. The prototype of the latter virus, named W1-PNENPO, expressed a small-plaque phenotype (Alexander *et al.*, 1994). However, translation of its RNA *in vitro* was identical to that of *wt* RNA (Alexander *et al.*, 1994). Our construct W1-PNENPO-2 (Fig. 1A2) was similar to W1-PNENPO except that 19 nt between the two IRES elements were deleted. RNAs derived from the plasmid DNA of PNENPO-2 or of dicistronic constructs in which a *wt* 3AB gene was inserted between the two IRES elements were translated *in vitro* using a HeLa cell-free extract (Molla *et al.*, 1991). The *wt* 3AB gene was constructed from the coding sequence of 3AB and was flanked at the N- and C-termini with initiation and termination codons, respectively. RNA of the *wt* poliovirus type 1 (Mahoney) [PV1(M)] was translated as a positive control. In order to differentiate between the 3AB polypeptides originating either from the first cistron or from the polyprotein, an *in vitro* translation kinetic experiment was performed. As shown in Fig. 2, translation patterns of wild-type RNA and PNENPO-2 RNA were identical throughout the time period from 30 min up to 8 hr at 34° (lanes 2, 8, and 14 for wild type; lanes 4, 10, and 16 for PNENPO-2). In contrast, the 3AB polypeptide originating from the first cistron in the dicistronic constructs DI3AB-1, -2, and -3 could be observed as early as 30 min after initiation of translation (lanes 5, 6, and 7). This observation indicated that the ORF of the helper 3AB gene was translated efficiently *in vitro* under the direction of the poliovirus IRES. Except for the early appearance of 3AB polypeptide, the patterns obtained from translation of DI3AB RNAs were nearly identical to that of *wt* RNA (Fig. 2).

Construction of the lethal VPg mutant pPVM(3F4A)

Our strategy to test for intragenomic complementation required that the mutation of the polyprotein-derived 3AB be lethal. That is, the genome must harbor a mutation(s) that abolishes genome replication; hence, no reversion or pseudoreversion to progeny virus can occur. Lethal mutations are contrasted with "transiently lethal" mutations, in which a very low level of RNA replication (usually escaping detection even by RT–PCR) may lead to reversion or pseudoreversion of the mutations at very low frequency (detected only if cultures are observed over extended periods of time). The resulting progeny viruses no longer harbor the original mutations. This phenomenon has been observed with many genetically engineered poliovirus genomes, and the phenotype has been referred to as "quasi-infectious" by Agol and his colleagues (Gmyl *et al.*, 1993). We will use this term throughout. Previously, a VPg mutation [VPg(Y3F)] had been constructed in which the tyrosine codon UAC was changed to UUU, a codon specifying phenylalanine. Full-length transcripts of pPVM-VPg(Y3F) carrying this mutation were found by dot-blot analyses to be RNA replication negative (Reuer *et al.*, 1990). However, we observed that the transcripts of pPVM-VPg(Y3F), when transfected onto HeLa cell monolayers and incubated for 5 days yielded in 10–20% of the experiments poliovirus growing with a *wt* phenotype. Sequence analysis of the progeny virus revealed reversion of the Phe UUU codon to a Tyr UAU codon. Apart from the unlikely possibility (see below) that a minute fraction of the pPVM-VPg(Y3F) construct contained a contaminating genotype with UAU in codon 3 of VPg, this result suggested that, astonishingly, the pPVM-VPg(Y3F) RNA was able to replicate, albeit at a very low level. It is, therefore, quasi-infectious. Low level replication of this quasi-infectious genome would eventually lead to the selection of a viable virus through a single point mutation. Although plausible, such explanation would hardly accommodate our model in which uridylylation of the O^4 hydroxyl group of the tyrosine residue is absolutely required for the initiation of RNA synthesis (Takeda *et al.*, 1986; Takegami *et al.*, 1983). We have, therefore, speculated that another amino acid in VPg could substitute for the lack of Tyr3. One candidate is a threonine in position 4 whose hydroxyl group could possibly function with very low efficiency as acceptor for the nucleotidyl group. It should be noted that serine residues are uridylylated in VPg of cowpea mosaic virus RNA (van Wezenbeek *et al.*, 1983) and deoxycytidylylated in the terminal protein of adenovirus DNA (Desiderio and Kelly, 1981; Smart and Stillman, 1982). Moreover, a VPg(3T4Y) mutant (pT7-VPg16), in which the H_2N-G–A–Y–T— sequence of VPg was changed to H_2N-G–A–T–Y— displayed detectable RNA synthesis although virus could not be recovered from this construct (Reuer *et al.*, 1990).

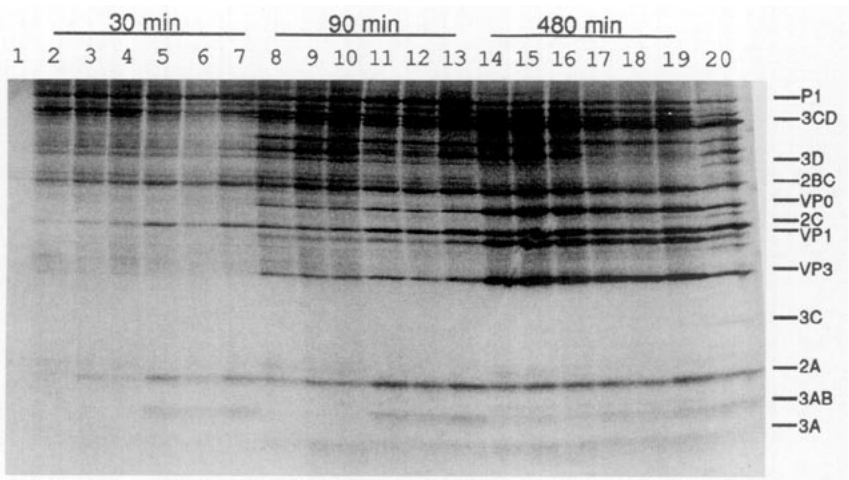

FIG. 2. *In vitro* kinetics of protein synthesis and processing directed by wild-type, VPg mutant, and dicistronic construct RNAs. *In vitro*-transcribed RNAs are translated in a HeLa cell extract from 30 to 480 min as indicated. The reaction mixtures were analyzed on an 10–20% gradient SDS–polyacrylamide gel. Lanes 2, 8, and 14, reactions with pT7-PVM RNA; 3, 9, and 15, reactions with pPVM-VPg(3F4A) RNA; 4, 10, and 16, reactions with pPNENPO-2 RNA; 5, 11, and 17, reactions with pDI3AB-1 RNA; 6, 12, and 18, reactions with pDI3AB-2 RNA; 7, 13, and 19, reactions with pDI3AB-3 RNA; 1, no RNA; 20, [^{35}S]methionine-labeled proteins of PV1-infected HeLa extract as the marker.

In order to test our hypothesis, we have constructed a cDNA clone pPVM-VPg(3F4A) that carried a double amino acid substitution at positions 3 and 4 of the VPg coding region, changing the tyrosine and threonine residues to phenylalanine and alanine, respectively. Translation of the VPg(3F4A) mutant RNA in the HeLa cell-free extract displayed translation patterns similar to that of *wt* RNA (Fig. 2, lanes 3, 9, and 15). Transfections of the double mutant RNA onto HeLa monolayers or COS-1 cells produced no cytopathic effect (CPE) after 5 days. No revertants could be recovered when RNA-transfected cells were lysed by cycles of freezing and thawing followed by incubation of the extract with the fresh HeLa or COS-1 cells. Moreover, synthesis of minus-strand RNA from transfections with this mutant RNA could not be detected using RT–PCR technique (data not shown). We conclude that VPg mutant pPVM-VPg(3F4A) was lethal, displaying a null phenotype. Viral genomes containing the VPg(3F4A) mutation were therefore used in our intragenomic complementation study, and they served also as negative controls in cell transfections.

Rescue of a lethal VPg(3F4A) mutation in a dicistronic virus

A *dc* genome was constructed containing two 3AB ORFs as indicated in Fig. 1A3. The first 3AB constitutes an independent cistron and can be considered *wt*, although the amino acid sequence of its 3B component is slightly different from that of VPg of PV1(M) in that the lysine residue in position 10 has been changed to an arginine (Fig. 1B; Kuhn *et al.*, 1988a). A poliovirus with the K10R mutation in VPg (derived from pT7-VPg15) has no detectable phenotype (Cao *et al.*, 1993; Kuhn *et al.*, 1988a). The second coding sequence of 3AB resided, naturally, in the polyprotein. It had either the *wt* (3Y4T in DI3AB-1; Fig. 1A3), or the lethal mutant sequence (DI3AB-2, -3; Figs. 1A4 and 1A5).

Transfection of HeLa monolayers with DI3AB-1 RNA led to complete CPE 30 hr post-transfection at 37°, about 5 hr later than transfection with PNENPO-2 RNA. The specific infectivity of PNENPO-2 and DI3AB-1 RNAs was about 10^3 PFU/μg, whereas that of PV1(M) RNA averaged 2×10^4 PFU/μg. Plaque sizes of viruses generated from PNENPO-2 RNA transfections were small compared to the plaque size produced by wild-type RNA, and the plaques from DI3AB-1 RNA were even smaller (Figs. 1A1 to 1A3). In contrast, transfection of HeLa cells with DI3AB-2 RNA produced little, if any, CPE after 4 or 5 days of incubation at 37°. However, when cells from DI3AB-2 RNA transfections were lysed and incubated with fresh HeLa cells, a small plaque virus (W1-DI3AB-2) was produced (Fig. 1A4). Passage of W1-DI3AB-2 led to the appearance of larger plaques that gradually became the dominant species after the fourth passage (Fig. 1A4). In contrast, plaque sizes increased much faster with W1-PNENPO-2 and W1-DI3AB-1, and the large-plaque variants were already dominant after the second passage (Figs. 1A2 and 1A3). All variants displayed plaques slightly smaller than the wild-type virus. It should be noted that when RNA of mutant VPg(3F4A) was used as negative control in all transfections and reinfections, CPE has never been observed.

In order to identify the genotypes of the recovered viruses, a series of RT–PCR experiments were performed. Using the oligonucleotide pairs 3115 and 3070 as primers, a 385-bp RT–PCR product was produced, an observation showing that the first cistron containing the 3AB *wt* gene in the first passage DI3AB-1 and DI3AB-2 RNAs was intact (Fig. 3A, lanes 3 and 5). Sequence analysis of the VPg region in the polyprotein revealed that,

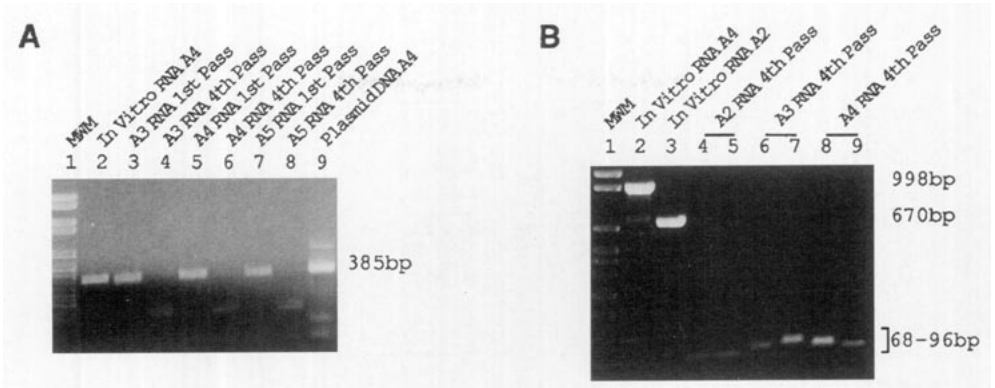

FIG. 3. RT–PCR analysis of the dicistronic viral RNAs isolated from infected cells. (A) Primers 3070 and 3115 (lanes 2 to 6 and 9) or 3070 and C14 (lanes 7 and 8) are used for the RT–PCR reactions for detecting the 385-bp band which represents the intact 3AB region at the 5′-end of the genome. A3, pDI3AB-1; A4, pDI3AB-2; A5, pDI3AB-3. (B) Primers 3070 and 3539 are used for the RT–PCR reactions. The 998- and 670-bp bands represent the intact genomes of plasmids pDI3AB-2 (lane 2) and pPNENPO-2 (lane 3), respectively, and the small bands with size 68–96 bp represent the deleted viral genomes derived from pPNENPO-2 (lanes 4 and 5), pDI3AB-1 (lanes 6 and 7), and pDI3AB-2 (lanes 8 and 9). MWM, molecular weight markers.

surprisingly, the lethal 3F4A mutation had reverted to the *wt* genotype (yielding a 3Y4T sequence). We conclude that the DI3AB-2 RNA is quasi-infectious, that is, even in the presence of intragenomic complementation, the RNA replicated to levels too low to form progeny virus. As will be shown below, variants carrying the sequence 3Y4A *in VPg are viable. The reversion to the wt sequence in* VPg of the polyprotein, therefore, is likely to have occurred by homologous recombination, involving an event of double cross-over (see below), rather than by multiple reversions of nucleotides (de la Torre *et al.,* 1992).

On the fourth passage DI3AB-1 and -2 viruses, the 385-bp band that was indicative of the first cistron had disappeared (Fig. 3A, lanes 4 and 6). Further RT–PCR analyses suggested that the entire 5-NTR of poliovirus was retained in the viral genome regardless of the number of passages (data not shown). The heterologous EMCV IRES, on the other hand, was deleted in all progeny virus after passage of the variants. As shown in Fig. 3B, a short band, averaging 80 bp in size, was the major RT–PCR product using oligonucleotide pairs 3539 and 3070 as primers (lanes 4 to 9). This result suggested that the EMCV IRES together with the first 3AB cistron were deleted in variants after the fourth passage.

Four well-isolated, large plaques of variants derived from *PNENPO-2, DI3AB-1,* and *DI3AB-2* RNAs were picked and expanded. The genomic RNAs of these variants were purified and directly sequenced using oligonucleotide 3539 as a primer. The results are shown in Fig. 4. As already found by RT–PCR analysis, all variant genomes had lost the EMCV IRES and 3AB gene, but the *deletions were not exactly the same. Interestingly, the* deletions observed in variant RNAs derived from PNENPO-2, DI3AB-1, and DI3AB-2 are not random, but they fall into four classes (rV2, rV3, rV5, and rV6; Fig. 4A). Since the events of deletion are likely to be random with respect to the length of the excised RNA, the observed restriction in the genotypes probably reflects selection at the level of replication. This is supported by the finding that variants of DI3AB-1 fall into three of the four classes of deletion mutants (Fig. 4; rV2, rV5, and rV6). The poliovirus IRES remained intact in all deletion mutants rV2, rV3, rV5, and rV6, but the length of the "spacer" between IRES and initiating AUG codon (Jang *et al.,* 1990) varied (Fig. 4). *Sequencing of the coding regions of 3AB mapping to* the polyprotein in variants rV2, rV3, rV5, and rV6 revealed *wt* sequences in all cases, as expected from the analyses of first-passage virus. It is therefore likely that the genotype of the DI3AB-2 revertants rV3 and rV5 must be the result of complex recombinational events rather than multiple base reversions.

Rescue with a *dc* virus containing heterologous VPgs in the two cistrons

Our data have shown that the lethal mutation in VPg(3F4A) of the poliovirus polyprotein could be rescued *in trans* by providing *wt* 3AB in the *dc* genome DI3AB-2. On passage of DI3AB-2, the first *wt* 3AB cistron and the EMCV IRES are deleted. Of course, this deletion must be preceded by the genetic repair [VPg(3F4A) to VPg(3Y4T)] of the second-cistron mutant 3AB in the quasi-infectious RNA, presumably by double homologous recombination. We have recently shown that two tandemly arranged VPg sequences in the P3 region are unstable (Cao *et al.,* 1993) and that the 3C-proximal VPg is deleted in all offspring viral strains by homologous recombination and/or homologous loop-out deletion (Cao and Wimmer, in preparation). For these studies, *we have constructed a coding region for VPg that was* heterologous in nucleotide sequence but homologous in amino acid sequence (Fig. 1B). This genetic trick was designed to prevent genetic rearrangements requiring nucleotide homology (Cao and Wimmer, in preparation). Homologous recombination by copy choice is likely to occur only during minus-strand synthesis (Kirkegaard

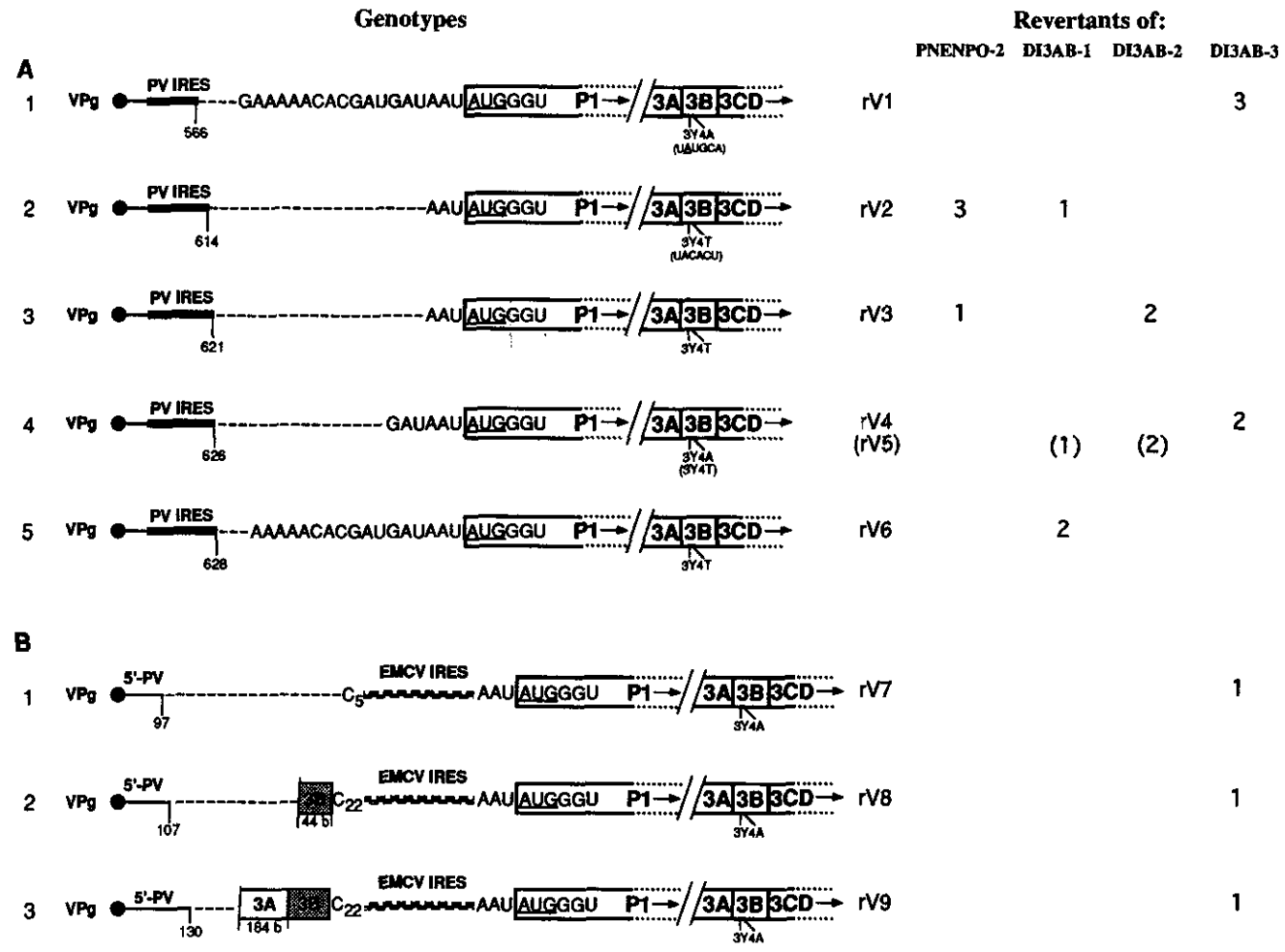

FIG. 4. Schematic representation of the revertant virus RNAs. Only the 5′-NTR and the amino acids at position 3 and 4 of the 3′-end VPg are shown. (A) All RNA contain only the poliovirus IRES element in the genome, but the "spacer" sequences from the 3′-end of the PV IRES to the initiated AUG codon are different. Positions of the 3′-end of the IRES are indicated (from 566 to 628). At the 3′-end VPg region, all the genomes containing the 3Y4A amino acids are encoded from the same nucleotide sequences (the U to A transversion is underlined). As control, the wild-type sequences 3Y4T are also indicated. (B) All RNA contain only the EMCV IRES element in the genome, but the linkage sequences between the 5′-end of poliovirus and the EMCV IRES are different. The positions of the 5′-end of the polioviral genome (from 97 to 130) and the length of the truncated 3AB region are indicated, all the 3′-end VPgs contain the 3Y4T amino acid sequences. "Spontaneously" deleted segments are denoted by dashes in both A and B.

and Baltimore, 1986), and that may also apply for homologous loop-out deletion (Cao and Wimmer, in preparation). Therefore, if the 3B (=VPg) region of the first cistron *wt* 3AB were heterologous in nucleotide sequence (Fig. 1B), repair of the second cistron mutant 3AB by recombination might not be possible. Accordingly, we have constructed a *dc* viral genome that is identical to DI3AB-2 except that the 3B region of the first-cistron wt 3AB had an altered nt sequence (DI3AB-3; Fig. 1A5; Fig. 1B).

Translation of DI3AB-3 RNA yielded a polypeptide pattern similar to that of the other two *dc* viral RNAs (Fig. 2, lanes 7, 13, and 19), an observation suggesting that translation and processing were not affected by the genetic alteration in DI3AB-3, as expected. HeLa cell monolayers transfected with DI3AB-3 RNA did not produce viable virus which were visible under an agar overlay (Fig. 1A5). However, when cells incubated for 4 to 5 days at 37° were lysed by cycles of freezing and thawing, and the cell extracts were used to reinfect fresh HeLa cell monolayers, virus expressing a minute plaque-size phenotype was observed. Continuous growth of this virus stock yielded viruses with a small-plaque phenotype, and this phenotype did not change significantly on fourth passage. As can be seen in Fig. 1A5, the plaque size of these W1-DI3AB-3 viruses was still smaller than that of W1-DI3AB-2.

To determine the genotypes of the W1-DI3AB-3 viruses, total viral RNAs purified from the first- and the fourth-passage virus were reverse-transcribed *in vitro* using oligonucleotides C14 and 3539 as primers, and the cDNAs were amplified by PCR. As with the DI3AB-1 and DI3AB-2 constructs, a 385-bp band was generated from primers 3115 and 3070 with first-passage viruses but this product had disappeared when fourth-passage DI3AB-3 viruses were analyzed (lanes 7 and 8 in Fig. 3A). Remarkably, the sequence of the VPg contained in the polypro-

tein had reverted to VPg(3Y4A) by a single nt change (UUU → UAU). This strongly suggests that no recombination had occurred to repair the lethal mutation in the second-cistron 3AB.

The nature of the small band of approximately 100 bp in length that appeared in analyses of all four passage RNAs is unknown (Fig. 3A, lanes 4, 6, and 8). It is not due to an internal deletion within the first 3AB cistron since the entire 3AB cistron had disappeared (Fig. 3B, lanes 6–9). RT–PCR analysis of the fourth-passage virus RNAs using primers C14, 2298, and 3539 indicated that the 5′-NTRs of these viruses were highly heterologous with respect to the length (data not shown). Eight viral RNAs of plaque-purified viruses from fourth-passage DI3AB-3 virus were directly sequenced using oligonucleotides 2298 or 3539 as primers. The results revealed remarkable heterology (Figs. 4A and 4B).

Several points are noteworthy. First, all VPg sequences in the polyprotein expressed the sequence VPg(3Y4A), an observation suggesting that the repair to the pseudorevertants occurred by misincorporation of nucleotides. Second, the deletions in these variants affected both IRES elements. Third, in some cases truncated 3AB ORFs of the first cistron were retained in the variants (rV8, rV9). Thus, neither the poliovirus IRES nor the EMCV IRES appears to provide a selective advantage for genome translation and replication. Fourth, two of the eight deletions in the 5′-NTR are identical (rV4) to those observed with DI3AB-1 and DI3AB-2 constructs (rV5). This implies that the rV4/rV5 genotypes compete favorably among other deletion products. Fifth, in variant rV1, not only the EMCV IRES but also a portion of the poliovirus IRES and most of the "spacer" has been deleted.

Plaque phenotypes of the revertants

The plaque sizes produced by variants rV2, rV3, rV5, and rV6 were only slightly smaller than those of *wt* poliovirus (data not shown) in spite of the deletions in the 5′-NTRs. This conforms to observations made previously in genetically engineered, or tissue selected, deletion mutants of poliovirus (Kuge and Nomoto, 1987; Pilipenko *et al.*, 1992). In contrast, all variants produced by DI3AB-3 RNAs produced medium (rV4) to small plaque sizes (rV1, rV7, rV8, and rV9) (Figs. 5B–5F). The growth properties of rV4 appeared to be slightly impaired compared to those of rV5 (data not shown), an observation suggesting that the Ala4 residue in VPg of rV4 is not optimal for 3AB and/or VPg function. The similarity of the plaque sizes of the other four variants (rV1, rV7, rV8, and rV9) suggests that the different genotypes of these genomes exert little effect on the overall proliferation of the viruses. Variant rV7 is interesting as the deletion of the poliovirus-specific sequences in the 5′-NTR extends almost to the border of the 5′-terminal cloverleaf, a structure essential for viral genome replication (Andino *et al.*, 1990, 1993; Harris *et al.*, 1994; Xiang *et al.*, 1995).

DISCUSSION

Genetic complementation of mutants mapping to VPg have not been described before, perhaps because suitable mutants were lacking. Attempts to generate VPg mutants with a conditional lethal phenotype, or with significant impairment of growth, have failed (Kuhn *et al.*, 1988a,b; Reuer *et al.*, 1990). The changes that have been introduced into the amino acid sequence of the VPg coding region either expressed a lethal phenotype or had little effect on virus proliferation. It must be emphasized, however, that many of the mutant genomes were probably quasi-infectious, that is, they allowed a very low level of genome replication which could eventually lead to the emergence of revertants. Indeed, this was also the case with the VPg(Y3F) mutation that, to our astonishment, yielded revertants after prolonged incubations of transfected cells. A previous report (Reuer *et al.*, 1990) claiming that a VPg(Y3F) mutation had a null rather than the quasi-infectious phenotype, is probably due to technical differences in the analyses. As discussed before, it is possible that the hydroxyl group of the threonine residue in position 4 of VPg may have been uridylylated at a very low level and that this nucleotidyl-VPg served in the initiation of RNA synthesis. In any event, although a VPg(T4A) mutation is tolerated by the virus (Fig. 4), the double mutation VPg(3F4A) is "absolutely" lethal, that is, long-term incubation of cells transfected with viral RNA harboring this double mutation has never yielded revertants in numerous transfection experiments. As expected, no viral RNA replication can be detected in these cells by RT–PCR. The null phenotype of genomes carrying VPg(3F4A) was useful for our studies of complementation since spontaneous reversions were highly unlikely to complicate the analyses.

We have designed our experiments to target polypeptide 3AB, the precursor for VPg, for complementation. Based on previous results by Alexander *et al.* (1994), we have constructed *dc* genomes to achieve simultaneous expression of two 3AB polypeptides from a single *dc* poliovirus genome. Whether necessary or not, this strategy assures that the two polypeptides would be synthesized in close proximity to each other, which may avoid problems of compartmentalization (Bienz *et al.*, 1990).

We have used the VPg(3F4A) mutation in the dicistronic genome that, under the conditions of our experiments, cannot yield viable virus by reversion. This lethal mutation, residing in the cistron encoding the polyprotein, can be rescued by *wt* 3AB expressed from a separate cistron, a phenomenon that can be accurately described as complementation. However, because the rescuing polypeptide is encoded in the same genome as the lethal polypeptide, we are describing a special case of complementation, for which reason we are strictly using the term "intragenomic complementation." Moreover, the rescuing gene product 3AB had to migrate, presumably by diffusion, to the site where 3AB is normally used.

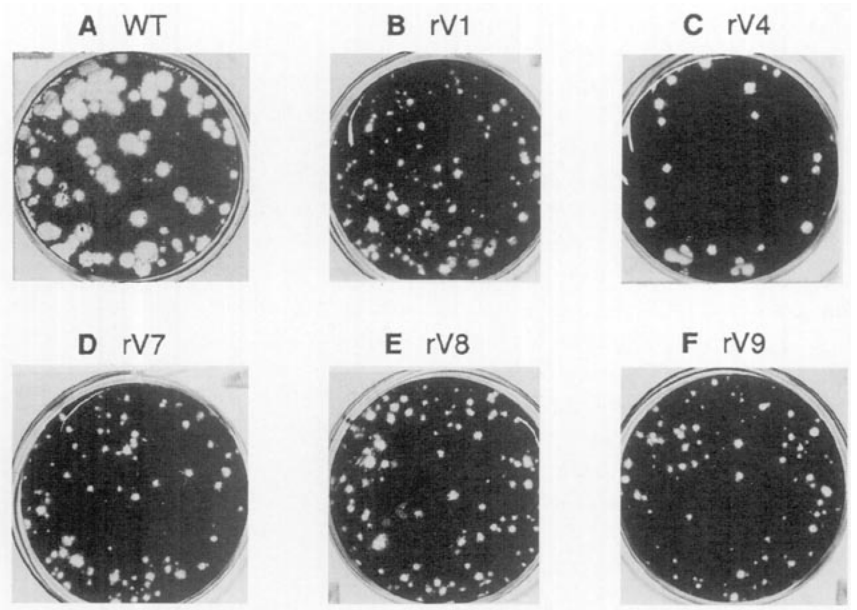

FIG. 5. The plaque-forming phenotype of the revertant viruses compared with that of wild-type PV1. The genome structures of the revertants are shown in Fig. 4. Plaques are developed at 48 hr postinfection.

Consequently intragenomic complementation must have occurred *in trans*. Whether a similar phenomenon of complementation can be observed when the rescuing polypeptide 3AB is provided from a separate genome is currently under investigation.

The intragenomic complementation that we have described here is very inefficient. In fact, the genomic constructs, in spite of replicating, express only a quasi-infectious phenotype. Considering recombination frequencies and the rates of misincorporation of nucleotides (Wimmer *et al.*, 1993), the replication machinery of the quasi-infectious RNA must synthesize several thousand progeny RNA strands allowing revertants to appear that, ultimately, may lead to virus formation (Gmyl *et al.*, 1993). In our case, the reversions mapped to the coding region for VPg. No second-site pseudoreverants were scored. In spite of a functioning VPg in the polyprotein, however, the first-passage dicistronic virus W1-DI3AB-2 was found to be genetically unstable. The reason for the low efficiency of intragenomic complementation of the VPg(3F4A) mutation is not known. 3AB has multiple functions in poliovirus genome replication (Harris *et al.*, 1994; Molla *et al.*, 1994; Paul *et al.*, 1994) and the mutant 3AB whose VPg portion carries the 3F4A sequence may be negatively dominant in some biochemical reactions. For example, on proteolytic processing, the polypeptide occurs in a complex with $3CD^{pro}$ (Molla *et al.*, 1994) or with $3D^{pol}$ (Paul *et al.*, 1994). If such complex is required for uridylylation, the exchange of $3AB^{mut}/3CD^{pro}$ to $3AB/3CD^{pro}$, or $3AB^{mut}/3D^{pol}$ to $3AB/3D^{pol}$, may be very inefficient.

The mechanisms by which the revertant genomes rV1–9 were generated are not known. It is likely that the repair of the double mutation 3F4A to 3Y4T in the rV2, rV3, and rV5 variants was the result of a double crossover during homologous recombination. This is supported by the observation that the introduction of a heterologous nt sequence in the 3B region of the first-cistron 3AB (DI3AB-3) had a dramatically delaying effect on the generation of viable progeny virus. Most importantly, the viable variants rV1, rV4, and rV7–9 of DI3AB-3 all reverted to 3Y4A by a point mutation and never to the *wt* sequence 3Y4T observed with the DI3AB-3 revertants rV2, rV3, rV5, and rV6 (Fig. 4).

Even more difficult to explain are the deletion events in the 5′-NTR leading to rV1–9. Strikingly, we observed a difference in the deletions of the IRES elements when the variants of PNENPO, DI3AB-1, and -2 were compared with those of DI3AB-3 (Fig. 4). The former did always delete the EMCV IRES and never the cognant poliovirus IRES (Fig. 4) although, admittedly, this may simply be a matter of the small number of samples that were analyzed. In any event, the unique genotypes of rV7–9 are intriguing. First, passage of viral genomes containing tandemly arranged IRES elements, such as W1-108ENPO or W1-PNENPO-2, has always yielded variants in which the heterologous (EMCV) IRES was deleted (Alexander *et al.*, 1994; Fig. 4). Isolates rV7–9 are the first naturally selected variants in which the heterologous IRES was retained. Second, the deletion of the poliovirus IRES and the retention of sizable segments of the 3AB ORF in the 5′-NTR (e.g., in rV9) argue against the possibility that interactions between cloverleaf and IRES play a significant role in translation and RNA synthesis. Third, it has been suggested recently that the poliovirus IRES may play an important role in poliovirus RNA replication (Borman *et al.*, 1994). If this were so then it is difficult to envision how the EMCV IRES can functionally replace

the poliovirus IRES in viruses rV7–9 or PV1(108ENPO) (Alexander *et al.,* 1994).

Finally, the genotype of rV1 is noteworthy. It has been observed previously that the 3′-border of all picornavirus IRES elements is formed by a Yn–Xm–AUG motif (Jang *et al.,* 1990; Pilipenko *et al.,* 1992) that has been shown to be functionally important for poliovirus and EMCV. In poliovirus, Yn of the motif maps to nt 559 to 563, and the (silent) AUG triplet to nt 581 to 583 (with Xm being 21 nt long). It is apparent that in rV1 the Xm–AUG portion of the motif has been deleted. However, a new motif was formed in which nucleotides from the EMCV IRES serve as Xm (m = 18) and AUG (Fig. 4, rV1). It should be noted that the EMCV-derived Xm sequence contains an AUG codon (AUG_{11} of the EMCV IRES) in excellent context for initiation of translation (**ACGAUGA**) just 5 nt upstream of the initiating AUG. Like in the EMCV IRES, this out-of-frame AUG is apparently ignored by the translational machinery (Jang and Wimmer, 1990; Kaminski *et al.,* 1994) or else its sequence would have been changed by mutation. This observation underscores the precision by which the translational machinery selects an initiating AUG codon in the context of an IRES. Very similar results have been obtained by Pilipenko *et al.* (1992), in the course of an extensive genetic analysis of the poliovirus Yn–Xm–AUG motif, and also by Haller and Semler (1992) in linker-scanning experiments. Moreover, Kuge *et al.* (1986) had previously shown that a substantial portion of the poliovirus 5′-NTR proximal to the ORF could be deleted *in vitro* without loss of virus viability; in this case, too, was a new Yn–Xm–AUG motif formed.

Although the genetic studies presented here deal only with 3AB, the strategy of intragenomic complementation that we have developed can be used in principle to study any other genetic unit of the poliovirus genome. Currently we are testing a mutant construct in which the lethal mutation maps to a different portion of 3AB.

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